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vector for delivery of a synthetic bovine growth hormone-releasing
factor gene into bovine cells.
SO JOURNAL OF ANIMAL SCIENCE, (1993 Mar) 71 (3) 687-93.

L4 ANSWER 49 OF 66 MEDLINE DUPLICATE 17
AU Kolb A F; Albang R; Brem G; Erfle V; Gunzburg W H; Salmons B
TI Characterization of a protein that binds a negative regulatory element in
the mammary-specific whey acidic protein
promoter.
SO BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, (1995 Dec 26) 217 (3)
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L4 ANSWER 50 OF 66 MEDLINE DUPLICATE 18
AU Petitclerc D; Attal J; Theron M C; Bearzotti M; Bolifraud P; Kann G;
Stinnakre M G; Pointu H; Puissant C; Houdebine L M
TI The effect of various introns and transcription terminators on the
efficiency of expression vectors in various cultured cell lines
and in the mammary gland of transgenic mice.
SO JOURNAL OF BIOTECHNOLOGY, (1995 Jun 21) 40 (3) 169-78.
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CHARACTERIZATION OF A PROTEIN THAT BINDS A NEGATIVE REGULATORY
ELEMENT IN THE MAMMARY-SPECIFIC WHEY ACIDIC PROTEIN PROMOTER

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Whey Acidic Protein (WAP) gene expression is restricted to the pregnant and lactating mammary gland. We have recently defined a negative regulatory element (NRE) in the WAP promoter which interacts with a factor (NBF) present in all nonWAP expressing cells (Kolb et al., 1994; J. Cell. Biochem. 56:245-261). Here we characterise this factor and show that although it is not related to a number of known transcription factors, including AP-1, NF-1 and SP-1, it may also be involved in controlling the expression from the mouse mammary tumour virus promoter. Three proteins that bind to the WAP-NRE have been identified, one of which is a 53kDa nuclear protein. This protein is present in nonWAP expressing cells, suggesting that it is responsible for limiting WAP expression to the pregnant and lactating mammary gland. This protein has been partially purified and its binding to the WAP-NRE is not appreciably affected by high salt concentrations. © 1995 Academic Press, Inc.

Rodent milk contains an abundant protein, whey acidic protein (WAP), the expression of which is limited to the mammary glands of pregnant and lactating but not virgin mice (Ref. 1 and references therein). The regulation of WAP gene expression is complex and involves pregnancy hormones as well as cell-cell interactions (2). Transgenic mouse studies have demonstrated that a 2.4kb WAP promoter fragment confers mammary specific expression

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upon a variety of linked heterologous genes during pregnancy and lactation (reviewed in 3). Thus the tissue specific and developmentally regulated expression of WAP is controlled, at least in part, by regulatory elements located in the 5'proximal sequences of the WAP gene.

Tissue specific expression of genes can be achieved by limiting the availability of positively acting transcription factors to the relevant cell types. WAP gene expression seems to be regulated by both hormone dependent and independent mechanisms. The concerted action of the hormones insulin, hydrocortisone and prolactin is required for WAP expression in mammary gland derived organ culture systems (4). More recently, hormone response elements have been identified in the mouse (5) and rabbit (6) WAP promoters, albeit in different locations, in transfection studies. Glucocorticoid hormones appear to indirectly affect transcription from the WAP promoter by inducing alterations in chromatin structure (7). The effects of prolactin also seem to be indirect in that prolactin induces a factor termed mammary gland factor (MGF) which acts synergistically with glucocorticoids to activate milk protein gene expression (reviewed in 8). Lactogenic hormones alone are however presumably not sufficient for the induction of WAP expression in the mammary gland since many other organs and cell types respond to pregnancy hormones but yet are not able to express the WAP gene (9). A number of binding sites for the transcription factor NF-1 are present in the WAP promoter and at least one of these has been shown to play an important role in the expression of the rat WAP gene (10). Other non-hormonal factors and enhancer responsive elements that may be involved in the regulation of WAP promoter activity have been identified (11, 12). One such binding site that binds mammary cell activating factor (MAF; Fig. 1) mediates hormone-independent mammary cell expression (13) and has been shown to be related to the Ets family of transcription regulators (14).

It is being increasingly recognised that the specificity of gene expression can also be regulated by factors that repress gene expression in all cells where expression is not required (for a review see 15). Recently we have identified a negative regulatory element (NRE) in the WAP promoter that may be a determinant of mammary specific expression (16). This WAP-NRE interacts with a nuclear protein (NBF; NRE binding factor) that is abundant in all cell lines and tissues examined in which the WAP gene is not expressed. Lactating mammary gland did not appear to express this protein as judged by band shift experiments (16). Here we characterize the proteins that interact with this WAP-NRE and show that a 53kDa protein present in nonWAP expressing cells, but not in pregnant or lactating mammary gland, expressing cells binds to the NRE. The interaction between this 53kDa protein and the WAP-NRE may thus be determinants of the mammary specificity of expression displayed by WAP. Further we show that NBF is not related to NF-1, but may be related to a factor that binds to the promoter of a mammary selective retrovirus.

MATERIALS AND METHODS

Band retardation analysis of DNA-protein interactions. Crude nuclear extracts were prepared (17) and total protein concentrations were determined by Bradford assay (Bio-Rad). Different amounts of these extracts were preincubated at room temperature with varying

concentrations of nonspecific competitor DNA (double stranded poly dIdC, Sigma) in 10mM HEPES pH 7.9, 50mM NaCl, 5mM MgCl₂, 10% glycerol, 2mM DTT for 15 min. In all band shifts a ³²P-ATP endlabeled WAP promoter XhoI/XbaI DNA fragment (Fig. 1) was added, the samples incubated at room temperature for a further 25 min to allow binding to occur and then separated on a native 6% polyacrylamide gel in a Tris-Borate-EDTA buffer system. The radioactively labeled DNA and DNA-protein complexes were visualized by autoradiography against X-OMAT (KODAK) film.

South-Western blot analysis of DNA-protein interactions. Nuclear proteins, prepared as described above (17), were separated on a 12% SDS polyacrylamide gels and transferred to nitrocellulose by a semi-dry electroblotting procedure (18). The transferred proteins were renatured on the nitrocellulose filter by incubation in 10mM Tris-HCl pH 7.4, 0.1mM EDTA, 0.05% Tween 20 for 20 min at room temperature. The filter was then incubated with 5 ng (50000 cpm) of a ³²P-ATP endlabeled DNA fragment in a hybridization buffer containing 10mM Tris-HCl pH 7.4, 0.1mM EDTA for 2h. After two 45 min. washes in the hybridization buffer at room temperature, the filters were exposed against X-OMAT film (KODAK).

Anion-exchange purification. Nuclear protein extracts (17), were applied Mono Q anion exchange column and the column washed extensively with 0.01M sodium phosphate, pH7.0. The negatively charged proteins that had bound to the positively charged matrix were then eluted with wash buffer containing increasing concentrations of NaCl.

RESULTS

Band shift competition assays

The protein that binds to the WAP-NRE, NBF has previously been shown to be present in nuclear extracts prepared from nonWAP expressing cell lines and organs but not in extracts from pregnant and lactating mammary glands (16). It has been reported that mammary specific genes, including WAP, contain binding sites in their promoters for AP-2 related factors (19) and NF-1 (10, 20, 21). To determine whether NBF is related to an already identified and well characterized transcription factor, band shift experiments were performed using, as specific competitors, double stranded unlabeled oligonucleotides containing the AP1, AP2, AP3, SP1 or NF1 binding sites (Fig. 2). The specific binding of NBF to the XhoI/XbaI fragment was not influenced by addition of an excess of unlabeled oligonucleotide containing the AP1 (Fig. 2, lane 4), AP2 (lane 5), AP3 (lane 6), Sp1 (lane 7), NF1 (lane 8) binding sites, suggesting that the CK protein interacting with the NRE is not related to any of these factors. The interaction of the NRE with nuclear proteins from cells that do not express WAP could, however, be competed by addition of an excess of an unlabelled 0.6kb PstI fragment from mouse mammary tumour virus (MMTV), a retrovirus that is preferentially expressed in the mammary gland of pregnant and lactating mice (22). This DNA fragment contains the proximal promoter sequences of MMTV (23)(Fig. 2, lane 9). The ability of this fragment to compete for NBF binding indicates that the promoter of this mammary specific retrovirus interacts with the same nuclear factor. In contrast addition of an excess of another MMTV DNA fragment carrying distal promoter sequences (0.9kb PstI; (23)) did not compete for binding of nuclear proteins to the WAP-NRE (Fig. 2, lane 10).

As expected, the characteristic NBF band shift is lost when nonlabeled WAP-NRE containing XhoI/XbaI fragment is used as a competitor (Fig. 2, lane 3).

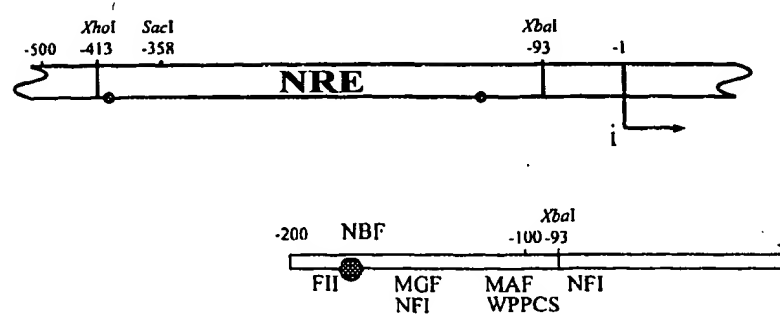


Fig. 1. Structure of the proximal region of the WAP promoter. Important restriction sites are indicated relative to the transcription initiation site (i, -1). A 320bp XhoI/XbaI restriction fragment (-413 to -93) mediates the negative regulatory effect (NRE) and is required in its entirety to obtain the band shift. Subfragments of this region no longer act as an NRE and are not shifted by NBF (16). The position of two binding sites for NBF are shown as shaded circles. The proximal region of the WAP promoter is shown enlarged below. As well as the proximal binding site for NBF (shaded circle), the binding sites for the transcription factors FII, MGF, MAF, WPPCS are shown (see (16) for exact location) as well as two binding sites for NFI. Potential glucocorticoid response elements are numerous and thus not indicated but can be found in (10).

WAP-NRE/NBF interaction is not appreciably affected by salt concentrations

The band shifts shown in Figure 1 and 2 were performed in a reaction buffer containing 50mM NaCl. The ability of a protein to bind to DNA is reduced by increasing salt concentrations. This provides a measure of the affinity of a protein for its recognition sequence and may be useful for the purification of the protein. The characteristic NBF shift was observed when 400mM NaCl was included in the binding reaction (Fig. 3, lane 2). Increasing amounts of NaCl resulted in a reduction in the proportion of free DNA shifted, but the characteristic shift was still detected, albeit weakly, even when 700mM NaCl was included in the reaction (lane 5).

Partial purification of NBF

As demonstrated above, NBF binds well to the WAP-NRE in the presence of 400mM NaCl. We attempted to purify NBF on the basis of this finding. Nuclear protein extracts from nonWAP expressing CK cells were applied to an anion exchange (MonoQ) column and step-wise eluted with a buffer containing increasing concentrations of NaCl. Each fraction was then analysed in band shift reactions for the characteristic NBF binding. The major NBF binding activity was present in the 300-400mM NaCl fraction (Fig. 4 cf. lanes 1&9). Fraction 9 (lane 10), and to a lesser extent in fraction 10 (lane 11), also contains significant binding activity, but the mobility of this shift is slightly different to the NBF shift (see discussion).

South-Western blot analysis of DNA-protein interactions.

The protein(s) binding to the XhoI/XbaI WAP-NRE fragment were characterized by South-Western blotting using a ^{32}P end-labeled XhoI/XbaI fragment as a probe. A limited number of nuclear proteins are able to bind the XhoI/XbaI fragment (Fig. 5, P1-53kDa; P2-

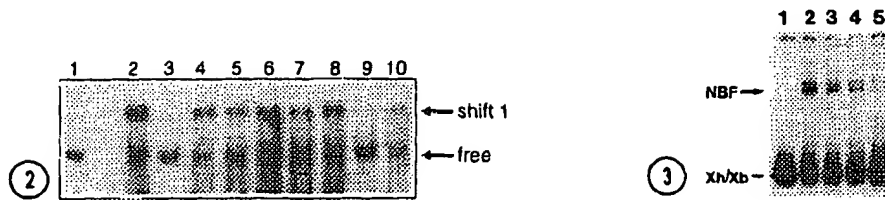


Fig. 2. Band retardation-competition analysis. Endlabeled XhoI/XbaI fragment was incubated with CK nuclear extracts and the nonspecific competitor poly dIdC, as previously described, in the absence (lane 2) or the presence of 25ng cold competitor XhoI/XbaI fragment (lane 3), 25ng of cold competitor synthetic oligonucleotide containing the binding site for AP1 (lane 4), AP2 (lane 5), AP3 (lane 6), SP1 (lane 7), NF1 (lane 8), or 200ng cold competitor 0.6kb MMTV LTR PstI fragment (lane 9) or 0.9kb MMTV LTR PstI fragment (lane 10). Lane 1 contains a control reaction without nuclear proteins. The positions of the free and retarded band (shift 1) are indicated.

Fig. 3. Effect of NaCl concentration on the WAP-NRE/NBF interaction. Band shift reactions were performed using the endlabeled XhoI/XbaI fragment without protein extract (lane 1) or with protein extract in binding buffer containing 400mM (lane 2), 500mM (lane 3), 600mM (lane 4) and 700mM (lane 5) NaCl as previously described. The position of the free XhoI/XbaI fragment (Xh/Xb) and of the shifted WAP-NRE/NBF complex (NBF) are indicated.

43kDa; P3-30kDa). A prominent protein of around 53 kDa (P1) is detected by the XhoI/XbaI fragment in extracts isolated from nonWAP expressing cells (Fig. 5, lane 1). This protein is also detected in nuclear extracts from a variety of other cell lines (e.g. Rat-2, GR, EF43, NIH3T3, and XC; not shown) which were previously shown contain NBF activity and are unable to express WAP (16). A protein of similar size could not be detected when nuclear extracts isolated from lactating or pregnant mammary glands were probed with the

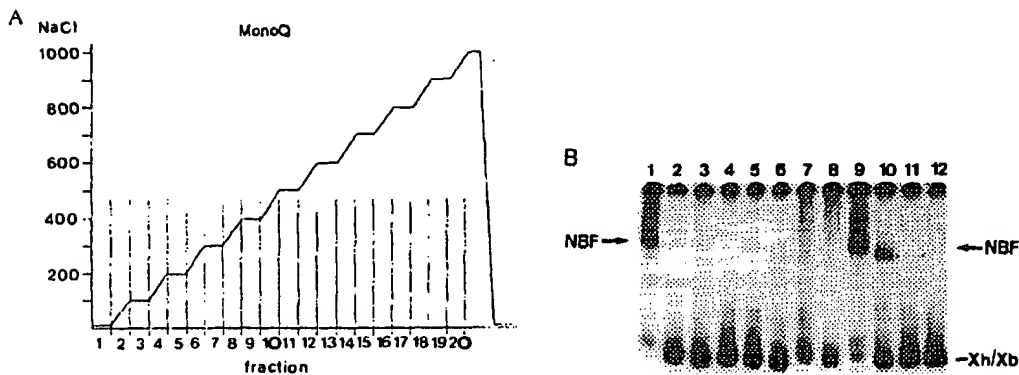


Fig. 4. Anion exchange column partial purification of NBF. (A) Profile of NaCl elution of proteins from the MonoQ column. The Y axis shows the concentration of NaCl in mM used to elute proteins from the column. (B) Band shift analysis of NBF activity in the fractions 1-11 from the MonoQ column (lanes 2-12), using the endlabeled XhoI/XbaI fragment as previously described. Nuclear protein extract prepared from CK cells and used directly in the band shift is shown in lane 1.

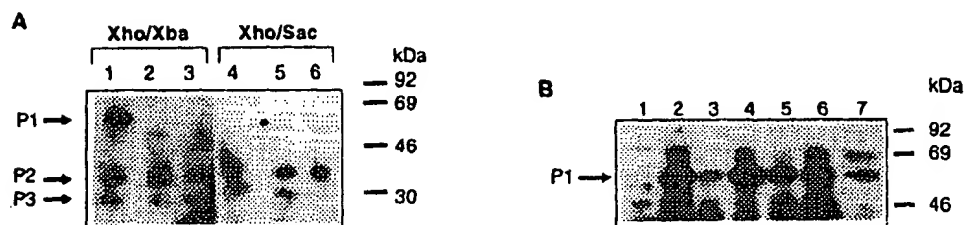


Fig. 5. South-western blot analysis (A) 40 μ g of crude nuclear extracts from CK cells (lanes 1 and 4), and from lactating (lanes 2 and 5) or pregnant (lanes 3 and 6) murine mammary gland were probed with a 32 P end-labeled XhoI/XbaI (lanes 1 to 3) or XhoI/SacI (lanes 4 to 6) fragment (Fig. 1A). A 53kDa protein that is present in CK cells, but not in the lactating or pregnant mammary gland, and that is detectable only with the complete XhoI/XbaI fragment is marked P1. Two other proteins P2 (43kDa) and P3 (30kDa), that are present in all nuclear extracts and can bind both the XhoI/XbaI and the XhoI/SacI fragment are also marked. (B) 40 μ g of crude nuclear extract prepared from lactating mammary gland (lane 1), Rat-2 (rat fibrosarcoma) cells (lane 2), NIH3T3 (mouse fibroblast) cells (lane 3), XC (rat fibrosarcoma) cells (lane 4), EF43 (mammary progenitor) cells (lane 5), GR (mammary tumour) cells (lane 6) and CK cells (lane 7) were probed with the 32 P end-labeled XhoI/XbaI fragment. A 53kDa protein that is present in all non-WAP expressing cells (CK, GR, EF43, XC, NIH3T3 and Rat-2) but not in lactating mammary gland (lane 1) is marked P1.

XhoI/XbaI fragment (Fig. 5, lanes 2 and 3 respectively). The 53kDa protein does not bind an end-labeled XhoI/SacI subfragment of the WAP-NRE (Fig. 5, lanes 4, 5 and 6). This subfragment, in contrast to the complete WAP-NRE, has been shown to be unable to confer transcription repression upon heterologous promoters (16). Thus the 53kDa protein is a candidate for the NBF activity described previously. In accordance with both the binding and biological properties of this protein, it does not seem to be present in extracts from lactating or pregnant mammary gland, but is present in a variety of nonWAP expressing cell lines.

In contrast two proteins of 43kDa and 30kDa that are present in mammary gland extracts as well as in CK extracts are detected upon hybridization of the nitrocellulose filters with the XhoI/XbaI fragment (Fig. 5, lanes 1, 2 & 3) as well as with the XhoI/SacI subfragment (lanes 4, 5 and 6). Further, the 30kDa and 43kDa proteins are detected in all extracts tested, regardless of whether they are isolated from nonWAP expressing cells and tissues or WAP expressing mammary glands. This binding to the XhoI/SacI subfragment of the WAP-NRE does not correspond to any functional property detected in previous expression analyses (16). No specific binding, however, could be detected when respective filters were probed with an end labeled SacI/XbaI fragment (data not shown). Binding reactions performed in the presence of cold specific XhoI/XbaI competitor lead to the loss of all signals (data not shown).

DISCUSSION

A number of cell types other than mammary epithelial cells respond to pregnancy hormones but do not express the WAP gene. This suggests that transcription regulatory mechanisms exist that prevent the expression of WAP in non-mammary cells, thereby also

contributing to the control of the tissue specific expression of this gene. Previously we have identified a binding site (NRE) in the 5' non-coding region of the WAP gene that represses transcription from linked heterologous promoters. This negative regulatory element (NRE) is located between -92 and -411 upstream of the WAP transcriptional initiation site and interacts with a nuclear protein, NBF, present in all nonWAP expressing cells and tissues examined. The interaction appears to suppress WAP expression in non-mammary cells (16).

NBF is able to interact with the WAP-NRE in conditions of relative high salt concentration- 400mM still gave appreciable binding which was diminished, though not abolished at higher concentrations. This property was exploited for purification of NBF on an anion exchange column. The major NBF containing fraction was eluted by 300-400mM NaCl and represents a purification of around 8 fold. If NBF is present in levels typical of other transcription factors such as SP-1, it would be necessary to start with 3g of protein extract and achieve 6000 fold purification (24). Interestingly, a slightly different band shift was observed after elution with 400-500mM NaCl (Fig. 4; starred, lanes 10 and 11). This lower mobility shift could be due to the loss of a protein from the NBF complex.

Southwestern analysis (Fig. 5) reveals that a number of proteins directly interact with the previously defined NRE. Nevertheless, one of these proteins, with a molecular weight of around 53kDa, is found exclusively in extracts from nonWAP expressing cells and this protein is only able to bind to the complete WAP-NRE containing XhoI/XbaI DNA fragment (Fig. 1). This protein alone or in a complex with other proteins is a good candidate for the NBF binding activity that is observed in band shifts.

If a complex of proteins is involved in the binding, the transcription factors AP1, AP2, AP3, SP1 or NF1 are probably not directly involved in binding since oligos carrying consensus recognition sites for these factors are not able to efficiently compete for binding in band shifts (Fig. 2). A potential binding site for NF1 is located with the WAP-NRE sequences (Fig. 1). NF1 like factors have been implicated in transcriptional control of MMTV (20), a retrovirus that is preferentially expressed in the mammary gland (22) and β -lactoglobulin, the major whey protein in the milk of ruminants (21). A binding site for the AP-2 transcription factor is also present in the MMTV as part of an enhancer that is selectively active in mammary cells (19). Neither the consensus AP-2 binding site oligo or the 0.9kb PstI fragment from the distal MMTV promoter region that carries the AP-2 containing enhancer are able to compete for WAP-NRE binding by NBF. By contrast the 0.6kb PstI promoter proximal fragment of MMTV was able to compete in the band shift assay. This region carries a number of regulatory elements that have a negative effect upon transcription from the MMTV promoter (reviewed in 22). Deletion of this region from the MMTV promoter results in expression in a number of organs and cell types that do not normally express MMTV i.e. a loss of mammary specificity of expression. Although these elements are relatively well defined, little is known about the proteins that interact with them (22). The results presented in this paper suggest that similar NBF binding sites may be present in the 0.6kb PstI MMTV promoter fragment and may play a role in regulating the mammary gland specificity of expression displayed by MMTV. We are currently investigating this further.

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